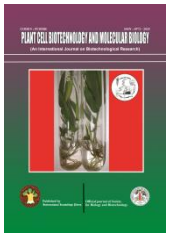

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Standardization of Protocol for Production of Synthetic Seed of Brahmi (*Bacopa monnieri*) and Assessment of their Longevity under Differential Storage Condition

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Brahmi [*Bacopa monnieri* (L) Pennel] is an important medicinal small succulent, medicinal herb belongs to the family 'Scrophulariaceae' which is commonly known as Jala Brahmi or Nira-Brahmi. National Medicinal Plants Board (NMPB), estimated an annual demand of Bacopa in 2004-2005 was 6621.8 tonnes, with a 7% annual growth rate. Micropropagation techniques and production of

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synthetic seeds can be a breakthrough to overcome the low availability of Brahmi propagating materials. So, the present study was conducted to standardize the protocol for preparation of synthetic seeds of Brahmi using nodal segment. The experiment was conducted with five treatments by using combinations of different concentrations of sodium alginate and calcium chloride and preparing two types of synthetic seeds (single layer and double layer). Further the longevity of both single layer and double layered synthetic seeds was studied under two different storage temperatures at 25°C (ambient condition) and 4°C (refrigerator) up to 40 days by inoculating them in two different types of media. The result showed no significant effect of concentrations of gelling agent and complexing agent on the germination percentage of synthetic seeds. The number of shoots per synthetic seed varied from 22.00 to 29.35 with 100% germination and maximum shoots/synthetic seed and shoot length was obtained by using T5 (single layered with MS media, 3% sodium alginate and 2.5% calcium chloride). Maximum number of days of storage can be observed in the single layer synthetic seeds when stored at 4°C along with media followed by double layer synthetic seeds when stored at 4°C along with media. However, seeds stored on vermiculite got dried up in the initial days of storage. Germination was found to be higher in seeds stored at 4°C when compared to seeds stored at 25°C. The study as a whole elucidated the opportunity for the use of synthetic seed as an alternative to traditional propagation system for Brahmi.

Keywords: Brahmi; micropropagation; synthetic seed; longevity.

1. INTRODUCTION

Bacopa monnieri (L) Pennel, commonly known as 'Brahmi' is an important medicinal small succulent, medicinal herb belongs to the family 'Scrophulariaceae' having chromosome number of 2n = 2x = 64 [1]. They grow at a temperature range between 33-40°C with relative humidity of 65-80% and it is grown mainly in humid and warmer regions of the world. It has been observed in wet and marshy areas and ascents to a height of 1320 m in various regions of India. It grows well in moist and wet soil. In India, it is found mainly growing in the states of Punjab, Uttar Pradesh, Bihar, Haryana, West Bengal, Kerala, Tamil Nadu, Karnataka and foothills of Himachal Pradesh and Uttaranchal. It is one of the most important top prioritized medicinal plants among 32 important medicinal plants in India identified for cultivation and conservation practices by National Medicinal Plants Board, New Delhi [2].

Each and every part of the plant can be used as medicine because it contains bio active constituents like bacoside A and B (including bacoside A1 and A3), tetracyclic triterpene saponins (jujubogenin and pseudojujubagenin), alakaloids (herpestine, nicotinin and brahmin), flavonoids (luteolin-7 glucoside, glucuronyl-7-apigenin, glucortonyl-7-luteolin and common phytosterols),glutamic acid, D-mannitol, stigmasterol, glycoside, cucurbitacin-E, cucurbitacinB, bittulinic acid, monnieraside I and III, and plantioside B, bacopasaponin A, B, C, D, E, and F, β-sitosterol, α-alanine, bacobitacin A, B, C, and D [3-11].

It is mainly propagated through stem cuttings or runners. Seed production of this crop is not found suitable for crop production. In view of these medicinal properties there is rapid increase in demand of the Bacopa based drug. According to the National Medicinal Plants Board (NMPB), the annual demand for Bacopa in 2004-2005 was 6621.8 tonnes, with a 7% annual growth rate. With the release of many new drugs based on this herb, there is going to be over exploitation of the natural resources of this herb which cannot meet the present requirement [12]. To prevent this medicinal herb from over exploitation and not going endangered, there is an immediate need to increase in production and conservation of this important germplasm.

Since seeds are short in availability and difficult to use as propagating material, micropropagation technique can be used as an alternative to fulfil the requirements [13]. 'Synthetic seed' is an important micropropagation tool for conservation and propagation of plant. Artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be utilised for sowing as a seed that has the capacity to develop into a plant under *in vitro* or *ex vitro* circumstances and that retains this potential even after storage are considered synthetic seeds [14]. To overcome low percent survival of *in vitro* plantlets during *ex vitro* acclimatisation, synthetic seed technology holds great promise because it allows the

vegetative propagule to be stored for long periods of time in complex mixtures and attempts to grow these synthetic seeds on different substrata while ensuring plant multiplication [15,16].

Although synthetic seeds have several advantages, including those considered for micropropagation like ease of handling, a high volume propagation system and reduced space requirements, however the storability of produced synthetic seed is an issue. Thus, the present study aims to standardize the method of synthetic seed preparation which can sustain its aliveness for a longer period.

2. MATERIALS AND METHODS

2.1 Materials

Local cultivar of Brahmi (*Bacopa monnieri*) was collected from Cooch Behar district of West Bengal, India. Initially the collected shoots of Brahmi were cultivated in pots (Fig. 1A). The shoots were collected from pots and inoculated on MS medium for micropropoagation (Fig. 1B&C). Young shoots with more number of nodes of Brahmi (*Bacopa monnieri*) were selected from *in vitro* grown and were cleaned properly using distilled water and surface disinfectant. They were treated with fungicides and then used for inoculation under aseptic condition into basal MS medium for establishment and incubated in a culture room at 25±2°C with 16/8 h light and dark phases.

Well grown *in vitro* plantlets of Brahmi were used as a source material of explants such as shoot tips and nodal sections.

2.2 Synthetic Seed Preparation

Synthetic seeds were prepared by encapsulating the propagating material with 3 percent and 5 percent sodium alginate solution and dropping into aqueous solution of 2.5 percent and 3 percent calcium chloride. Both the solutions were autoclaved at 121°C for 15-20 minutes at 15 psi pressure.

Well grown *in vitro* plantlets were selected for preparation of propagules. Nodal regions of well grown plantlets were selected and were trimmed under laminar air flow chamber and those nodal segments were used as a source of propagules for preparation of synthetic seeds. The nodal regions which were trimmed are mixed with sodium alginate solution. Individual propagule was transferred into calcium chloride solution with the help of a graduated dropper. After that calcium alginate beads were formed within 15-20 minutes. Beads were taken out by decanting the calcium chloride solution. Now the beads were washed with sterile double distilled water and surface of the seeds were dried with sterilized blotting paper. The surface dried seeds were inoculated into culture bottles.

Fig. 1. Establishment of culture. A) Locally collected shots were established in pot; B&C) *In vitro* established plants

2.3 Procedure for Preparation of Double Layer Synthetic Seeds

After the preparation of single layer synthetic seed, they were mixed again with sodium alginate solution containing fungicide Bavistin and they were transferred to calcium chloride solution with the help of dropper. Decanted the calcium chloride solution and wash the double layer beads with sterilized double distilled water and dried on blotting paper. The dried calcium alginate beads were inoculated into culture bottles.

2.4 Germination of Synthetic Seeds

The synthetic seeds were inoculated into two types of culture bottles *i.e*., culture bottle without media and culture bottle containing MS media fortified with 2 mg/L BAP and 1 mg/L kinetin. For each treatment 60 beads were employed and observations were recorded on 60^{th} day of inoculation. The experiment was conducted entirely under laminar air flow chamber.

2.5 Storage of Synthetic Seeds

After the preparation of single layer and double layer synthetic seeds they were stored at 25°C and 4°C temperature conditions in petri plates containing with media and without media and assessed for every 10 days of interval up to 40 days.

3. RESULTS AND DISCUSSION

Synthetic seeds which were produced from nodal segments of the *in vitro* mass-multiplied *Bacopa monnieri* (L.) with two different concentrations of both sodium alginate as gelling agent and $CaCl_2$ as complexing agent (Fig. 2A&B). Gelling agent was fortified with MS nutrient medium and control was taken without MS media. The seeds were inoculated on MS basal medium and different observations on quality parameters were taken on 60^{th} day of inoculation.

3.1 Germination of Synthetic Seed

Regeneration potential of synthetic seeds gives the assurance of success of the technique used. Here in the study, total 60 seeds were inoculated per treatment in replication (Fig. 2C&D). On 60^{th} day of inoculation, 100% germination was observed for all the treatments (Fig. 2E, F, G&H). The result indicated that there was no effect of the concentrations gelling agents and complexing agents on germination of synthetic seeds.

3.2 Number of Shoots per Synthetic Seed and Shoot Length

A good number of shoots and proper growth of shoot is required character. The number of shoots per synthetic seed showed significant variation among the treatments and it varied from 22.00 to 29.35 (Fig. 2 I,J&K, Fig. 2A). Maximum number of shoots per synthetic seed (29.35) were reported in T5 i.e., single layered synthetic seeds prepared with 3% sodium alginate, 2.5% $CaCl_2$ and fortified with MS medium.

Along with number of shoots, the shoot length also varied significantly among different treatments (Fig. 3B). Longest shoot length (6.22 cm) was observed in T5 followed by T3 (double layered synthetic seeds prepared with 5% sodium alginate, 3% $CaCl_2$ and fortified with MS nutrient media).

3.3 Number of Roots per Seed and Root Length

Number of roots per synthetic seed varied from 6.70 to 7.52 (Fig. 2.C). Maximum number of roots per synthetic seed (7.52) was observed in T5 followed by T1.

Similarly, the root length of synthetic seed also showed significant variations among the treatments. It varied from 7.52 cm to 5.14 cm (Fig. 2.D). Longest root was observed in T3 (Double layered with MS media, 5% sodium alginate, 3% $CaCl_2$) followed by T4 (single layered synthetic seeds prepared with 3% sodium alginate, 2.5% $CaCl_2$ and fortified with no MS nutrient media).

3.4 Assessment of Longevity of Synthetic Seeds

Longevity of synthetic seed important for conservation purpose as well as for transportation purpose. Having good longevity can be transported to long distance and also can be conserved the important genotypes. Many researchers stored encapsulated shoot tips and nodal segments at different temperature conditions [17,18,19]. The prepared synthetic seeds were conserved up to 40 days in two different storage temperature (25°C and 4°C) on two different media (MS and vermiculite) (Table 1 and Fig. 4). Initial viability was 100% for all the

seeds. The viability percentage changed with time. On 10th day of inoculation, 100% germination was recorded in seeds which were inoculated on MS basal medium under both the temperatures. However, all the seeds which were kept under vermiculite were dried.

Further reduction in germination was observed in seeds inoculated on MS media. More reduction in germination was observed in doubled layered synthetic seeds as compared to the single layered synthetic seeds. Germination was found to be higher when the synthetic seeds were stored at 4°C as compared to storage at 25°C (Table 1 and Fig. 4).

Brahmi (*Bacopa monnieri* (L) Pennall), is an important medicinal plant which were long been known traditionally and used in Indian Ayurvedic therapies for the treatment for mental illness, asthma, anxiety, and age-related, Antioxidant, Stress, cough and cold etc. [20]. To overcome the low availability of propagating material, the technique of synthetic seed can proof itself as an interesting and alternative approach. Synthetic seeds have been recently marked a breakthrough in *in vitro* culturing of plant cell tissue and have helped to overcome various challenges especially that face important economic and medicinal plant species [21]. The technique is a fine combination of micropropagation and encapsulation techniques. Synthetic seeds are alginate encapsulated somatic embryos, vegetative buds, or any other micro propagules that can be used as seeds.

In the present study five different compassions were used for preparation of synthetic seeds, namely double layered with MS media, 3% sodium alginate, 2.5% $CaCl_2$, double layered without MS media, 3% sodium alginate, 2.5% $CaCl_2$, double layered with MS media, 5% sodium alginate, 3% $CaCl_2$, single layered without MS media, 3% sodium alginate, 2.5% $CaCl_2$ and single layered with MS media, 3% sodium alginate, 2.5% $CaCl_2$. However, 100% germination was reported for all the treatments. The result indicated that there was no effect of the concentrations gelling agents and complexing agents on germination of synthetic seeds when it was inoculated on MS medium on the day of preparation of synthetic seeds.

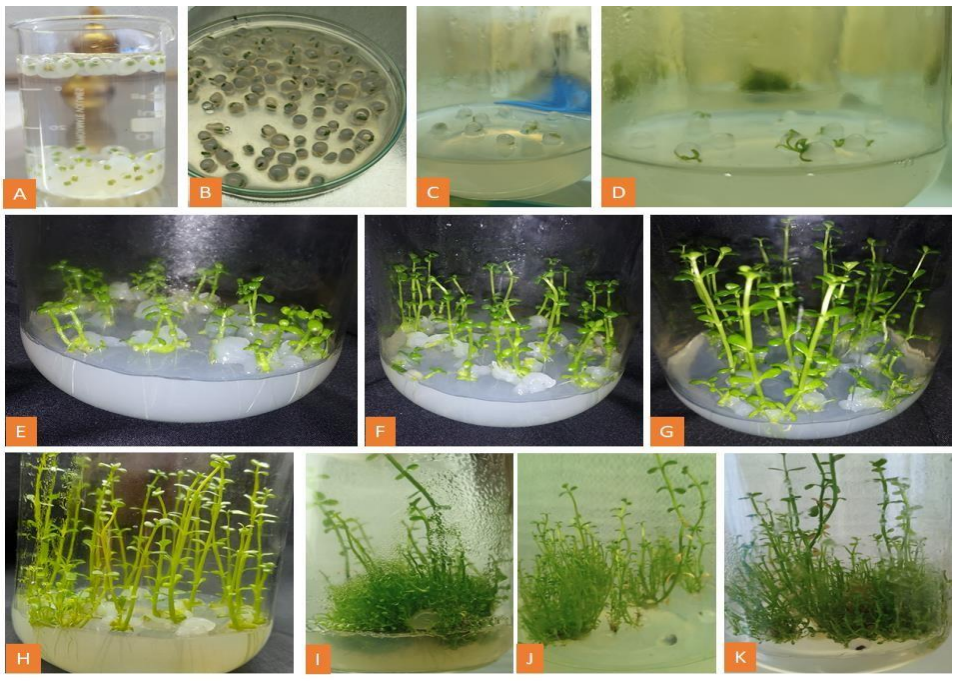

Fig. 2. Germination and establishment from synthetic seeds. A) Prepared synthetic seeds in $CaCl_2$; B) Synthetic seeds after decantation of $CaCl_2$; C) Inoculated synthetic seeds prepared from nodal segments; D) Inoculated synthetic seeds prepared from shoot tips segments; E) Germinated synthetic seeds prepared from nodal segments; F&G) Germinated synthetic seeds prepared from shoot tips segments; H) Grown up plantlets of synthetic seed prepared from nodal segments; I) Multiple shoots of synthetic seed prepared from shoot tips after 60 days of inoculation; J&K) Multiple shoots of synthetic seed prepared from nodal segments after 60 days of inoculation

Table 1. Performance of synthetic seeds on MS medium after different phases of storage

	Treatments					No. of synthetic seed germination				
Treatments	**No. of layer**	**Sodium alginate**	**$CaCl_2$**	**Medium**	**Storage Temp.**	**Day 0♣**	**Day 10**	**Day 20**	**Day 30**	**Day 40**
T1	Single*	3%	2.5%	MS	25°C	60	60	Dried	Dried	Dried
T2	Single*	3%	2.5%	MS	4°C	60	60	48	36	Dried
T3	Single*	3%	2.5%	Vermiculite	25°C	60	Dried	Dried	Dried	Dried
T4	Single*	3%	2.5%	Vermiculite	4°C	60	Dried	Dried	Dried	Dried
T5	Single**	3%	2.5%	MS	25°C	60	60	54	52	50
T6	Single**	3%	2.5%	MS	4°C	60	60	60	60	54
T7	Single**	3%	2.5%	Vermiculite	25°C	60	Dried	Dried	Dried	Dried
T8	Single**	3%	2.5%	Vermiculite	4°C	60	Dried	Dried	Dried	Dried
T9	Double*	3%	2.5%	MS	25°C	60	60	42	Shrunken	Dried
T10	Double*	3%	2.5%	MS	4°C	60	60	60	54	42
T11	Double*	3%	2.5%	Vermiculite	25°C	60	Dried	Dried	Dried	Dried
T12	Double*	3%	2.5%	Vermiculite	4°C	60	Dried	Dried	Dried	Dried
T13	Double**	3%	2.5%	MS	25°C	60	60	48	48	42
T14	Double**	3%	2.5%	MS	4°C	60	60	60	56	53
T15	Double**	3%	2.5%	Vermiculite	25°C	60	Dried	Dried	Dried	Dried
T16	Double**	3%	2.5%	Vermiculite	4°C	60	Dried	Dried	Dried	Dried
T17	Double*	5%	3%	MS	25°C	60	60	18	ψ	ψ
T18	Double*	5%	3%	MS	4°C	60	60	24	ψ	ψ
T19	Double*	5%	3%	Vermiculite	25°C	60	Dried	Dried	Dried	Dried
T20	Double*	5%	3%	Vermiculite	4°C	60	Dried	Dried	Dried	Dried
T21	Double**	5%	3%	MS	25°C	60	60	12	12	ψ
T22	Double**	5%	3%	MS	4°C	60	60	14	15	ψ
T23	Double**	5%	3%	Vermiculite	25°C	60	Dried	Dried	Dried	Dried
T24	Double**	5%	3%	Vermiculite	4°C	60	Dried	Dried	Dried	Dried

***: Synthetic seed without MS media; **: Synthetic seed with MS media*

ψ: Not germinated, but seeds are viable; ♣: Inoculation was done on MS basal medium without any storage

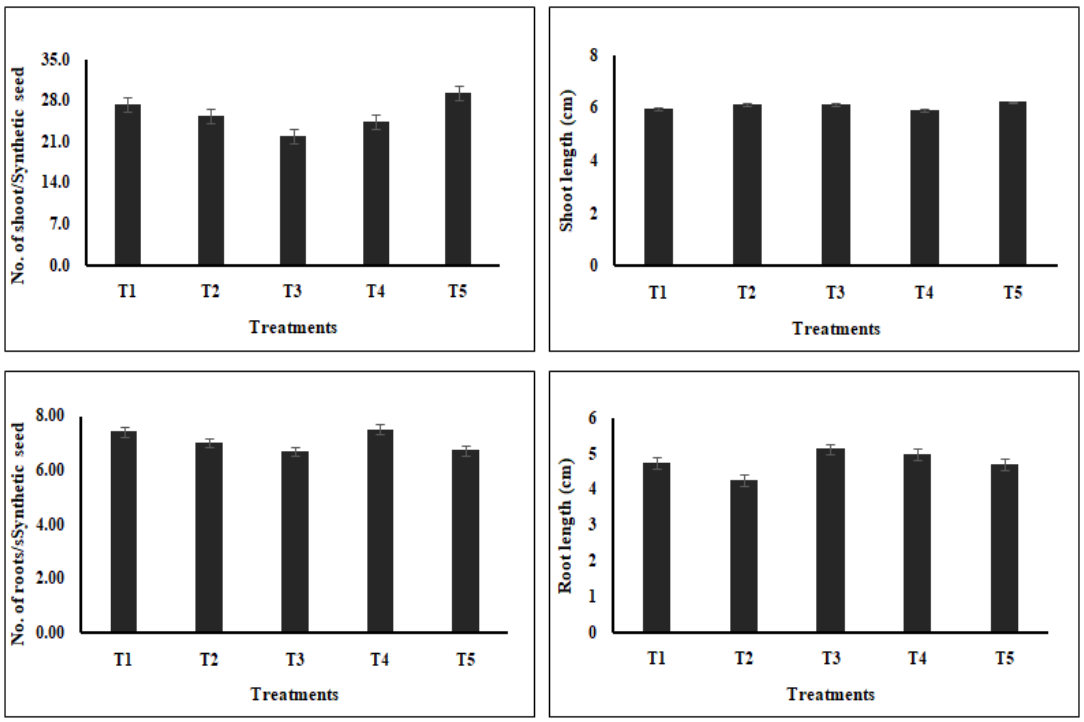

Fig. 3. Performance of synthetic seeds on 60th day of inoculation

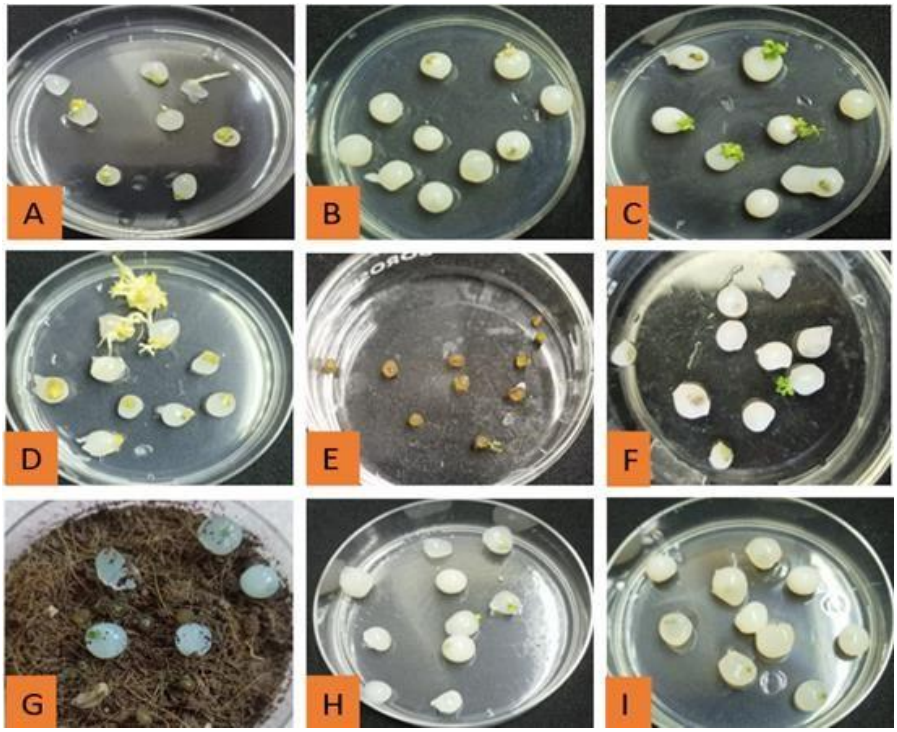

Fig. 4. Synthetic seeds storage. A) Single layer synthetic seeds stored without media at 4°C; B) double layer synthetic seeds stored without media at 25°C; C) Single layer synthetic seeds stored with media at 4°C; D) Single layer synthetic seeds stored at 4°C with media; E) dried seeds of single layer synthetic seeds stored at 25°C without media; F) double layer synthetic seeds stored at 25°C without media; G) double layer synthetic seeds kept on vermiculite for regeneration; H) shrunken seeds of double layer synthetic seeds stored at 25°C; I) double layer synthetic seeds stored at 4°C

However, single layered synthetic seeds performed better than the double layered synthetic seeds. When the concentration of sodium alginate increased (from 3% to 5%), the germination of the double layered synthetic seeds reduced remarkably. Micheli et al. [22] conducted experiment by double encapsulating the M.26 apple rootstock for conversion by comparing the double encapsulation with single encapsulation and encapsulation coating materials and concluded that the double encapsulation did not showed any detrimental effects on viability, sprouting and regrowth of the encapsulated micro cuttings and less than 40% of the synthetic seed had been converted into plantlets. Contrary, Maruyama et al. [23] reported higher germination of double layer artificial seeds of *Cedrela odorata* L., *Guazumacrinita* MART., and *Jacaranda mimosaefolia* D. DON.

Additional advantages of synthetic seed can be marked as its ease of preservation. So, encapsulated propagules may be used for germplasm preservation of elite plant species and exchange of plant materials between national and international laboratories. However, for this, the assessment of longevity is very important. In the present study, reduction in conversion of synthetic seed was observed along with the longevity of the storage. Synthetic seeds did not germinate on vermiculite and subsequently dried. Germination was lower in doubled layered synthetic seeds as compared to the single layered synthetic seeds. Germination was found to be higher when the synthetic seeds were stored at 4°C as compared to storage at 25°C.

4. CONCLUSION

The number of shoots per synthetic seed varied from 22.00 to 29.35 with 100% germination. The maximum shoots/synthetic seed, shoot length was obtained by using T5 (single layered with MS media, 3% sodium alginate and 2.5% calcium chloride). Maximum number of days of storage can be observed in the single layer synthetic seeds when stored at 4°C along with media followed by double layer synthetic seeds when stored at 4°C along with media. However seeds stored on vermiculite got dried up in the initial days of storage. Germination was found to be higher in seeds stored at 4°C when compared to seeds stored at 25°C. The study concludes that there is the opportunity for the use of synthetic seeds as an substitute to traditional propagation system for Brahmi.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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